

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph on page 3 of the specification (background), that begins on line 18, with the following amended paragraph:

It has been shown by recent studies (Biochem. Biophys. Res. Commun. 294: 934-939, 2002) that cell death, whether by necrosis or apoptosis, has orderly DNA fragmentation. Thus kilobase and 200 bp DNA ladder fragmentations obtained by way of necrosis from freezing, demonstrated a marked pattern of DNA base sequence selection, similar to that in apoptosis. The genomic GC clusters with high density were preferentially GCn*GC (SEQ ID NO: 1) motifs. They seem to align with metamer regularity in the genome of man to virus, and thus preserve a high degree of regularity in the chromatin conformation.

Please replace the last paragraph on page 3 (background), beginning on line 26 and going to page 4 line 8, with the following amended paragraph:

Necrosis from freezing manifested an orderly pattern of DNA fragmentations including the apoptosis signature of 200 bp ladder, in three different cell populations despite pancanapse suppression by zVAD-fmk. Immediately on thawing, all three populations had 100% dead cell indices and 2.2, 1.6, and 1.1 megabase fragmentations, which marked the point of death. Kilobase and 200 bp DNA ladder fragmentations manifested later together with overt necrotic morphologies. CpG oligodeoxynucleotides (ODNs) complementary to highly conserved GCnGC (SEQ ID NO: 2) motifs inhibited the megabase fragmentations and retarded their electrophoretic mobility (gel shift), indicating ODN-DNA binding, which is known to confer site-specific resistance to cleavage. Cleavage specificity was confirmed using EDTA-CpG ODN conjugates to direct free-radical-producing transitional element, vanadyl(4), to the binding sites to reproduce the megabase fragmentations in

normal cells. Specific orderly fragmentation in necrosis suggested a necrosis-apoptosis convergence after death has been committed (Biochem. Biophys. Res. Commun. 294: 934-939, 2002).

Please replace the paragraph on page 4 line 9 through line 17 , with the following amended paragraph:

Thus, in a recent study, it was shown that the complementary CGn*CG (SEQ ID NO: 3) oligodeoxynucleotide (ODN) sequences in parallel orientation were able to inhibit megabase DNA fragmentation, indicating ODN-DNA binding, and thereby conferring site-specific resistance to cleavage (Biochem. Biophys. Res. Commun. 294: 934-939, 2002). A series of ODN, with GCn*GC (SEQ ID NO: 1) motifs, where n= 2, 5, 9, were synthesized in this study. The test examples synthesized were 5'-GCnnGC-3' (SEQ ID NO: 4), 5'-GCnnnnnGC-3' (SEQ ID NO: 5), 5'-GCnnn nnn nnn GC-3' (SEQ ID NO: 6). These oligonucleotides, which are complementary to the highly conserved GCn*GC motifs, after conjugation inhibited the megabase fragmentations, thereby indicating ODN-DNA binding.

Please replace the last paragraph on pg. 13 (beginning on line 17 and going until page 14 line 13), with the following amended paragraph:

The studies of Dr. Kwok-Hung Sit and colleagues (Yee-Jiun Kok, Myint Swe, and Kwok-Hung Sit, Biochemical and Biophysical Communications, 294 934-939, 2002), suggest a relationship between the cell death and immunostimulatory activity. With the effective ODN-CpG binding, there is strong inhibition of CpG DNA fragmentation, resulting in site specific resistance to cleavage, and thereby prevent necrosis and apoptosis. CpG oligonucleotides referred in the preceding discussions, however, independently seem to enhance the immunostimulatory activity. On the other hand inhibition of megabase fragmentation of the highly conserved GCn*GC (SEQ ID NO: 1) motifs by

complementary ODN's will help to protect complementary CpG DNA from degradation. Our approach of ODN design and incorporation of potent anticancer drug is a novel approach and presents enormous future potential in molecular medicine. The incorporated anticancer drug can be cleaved by one of the several endolytic cleavage mechanisms. This should result in hydrolysis of a phosphodiester bond, esterase hydrolysis of the ester linkages outlined in the details of claims or amide hydrolysis will liberate the anticancer drug. While the CpG ODN will act as complementary DNA-ODN conjugate for the stability from degradation, it also seems that with proper design selection of the CpGn*CpG (SEQ ID NO: 3) ODN, immunostimulatory properties of the ODN could be available within the cell.

(Please replace the description of the figures (i.e., page 14, line 18 through page 15 line 6) with the following amended description; no amendments are required for the description of the figures on page 15 lines 7-13)

Figures

FIG. 1 shows the UV absorption spectra data of the oligonucleotide dFC GG ACG (SEQ ID NO: 7).

FIG. 2 shows the UV absorption spectra data of the oligonucleotide dFC GTG GAA CG (SEQ ID NO: 8).

FIG. 3 shows the UV absorption spectra data of the oligonucleotide dFC GGA CGT GGA ACG (SEQ ID NO: 9).

FIG. 4 shows the UV absorption spectra data of the oligonucleotide dFC GGA GCT GGA ACG (SEQ ID NO: 10).

FIGs. 5 A-B shows the HPLC of the sequence dFC GGA CG (SEQ ID NO: 7).

FIGs. 6 A-B shows the HPLC of the sequence dFC GTG GAA CG (SEQ ID NO: 8).

FIGs. 7 A-B shows the HPLC of the sequence dFC GGA CGT GGA ACG (SEQ ID NO: 9).

FIGs. 8 A-B shows the HPLC of the sequence dFC GGA GCT GGA ACG (SEQ ID NO: 10).

FIGs. 9 A-B shows the Capillary Gel Electrophoresis of the sequence dFC GGA CG (SEQ ID NO: 7).

FIGs. 10 A-B shows the Capillary Gel Electrophoresis of the sequence dFC GTG GAA ACG (SEQ ID NO: 8).

FIGs. 11 A-B shows the Capillary Gel Electrophoresis of the sequence dFC GGA CGT GGA ACG (SEQ ID NO: 9).

FIGs. 12 A-B shows the Capillary Gel Electrophoresis of the sequence dFC GGA GCT GGA ACG (SEQ ID NO: 10).

Please replace the paragraph on pg 16 line 11 through line 27 with the following amended paragraph:

A “modified phosphate” is a phosphate group of the formula R-P=O that is part of the internucleotide linkage between two nucleotides. Examples of modified phosphates of the invention include natural phosphodiester (R= OH), alkoxy phosphotriester (R is a lower alkoxy containing 1 to 6 carbon atoms such as OCH₃, OC₂H₅, n- OC₃H₇, that is, a straight chain n- OC₃H₇, or iso-OC₃H₇), a substituted lower alkoxy, such as 1-6 carbon, ~~but not limited to what(?)~~, as set forth in U.S. Pat. No. 5,023,243 and European Patent No. 0 092 574, both of which are incorporated by reference), phosphorothioate, (R = S; i.e., one of the non-bridging oxygens is replaced with sulfur, as set forth in International Patent Application WO 95/26204, herein incorporated by reference), and phosphoramidate (R= NH, as described in U.S. Pat. No. 4,469,863, which is incorporated herein by reference), and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and

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Peyman, A. Chem. Rev. 90:544, 1990; Goodchild, J. Bioconjugate Chem. 1:165, 1990. All references cited are incorporated herein by reference.).

Please replace the first paragraph on page 22 (line 5 through line 23) with the following amended paragraph:

The prodrug may also be linked by a 3'-5' linkage or a 5'-3' linkage, both of which are well known to the skilled artisan. Attachment of a prodrug to the oligo via a linker, will cause liberation of the prodrug at the cancer cell sites by hydrolytic enzymes and will have the effect of the prodrug as well as the CGn*CG (SEQ ID NO: 3) oligonucleotide. An example of a point of cleavage between a prodrug and the oligonucleotide is an ester linkage. The aliphatic esters are chosen for this purpose, since they are stable, yet can be easily hydrolyzed inside cells by intracellular esterases. Aliphatic phosphate esters, alkyl substituted phosphate esters, and amides are also part of this discovery, since they are also hydrolyzed by intracellular enzymes. Aliphatic amide linkages are chosen at the other side of linkage. The amide linkage is generally required in order to attach the prodrug and oligonucleotide. Thus, one end bears a carboxylic or activated ester of carboxylic acid, and the other end has a free amino function, to effect the joining of two moieties. This approach is extensively used in oligonucleotide labeling with various chromophores and ligands (See, P.S. Nelson, M. Kent and Sylvester Muthini, Nucleic Acids Research, Vol. 20, No.23: 6253- 6259, 1992; Misiura, K., Durrant, I., Evans, M.R., and Gait, M., Nucleic Acids Research, Vol., 18: 4345-4354, 1990; Zendegui, J.G., Vasquez, K.M., Tinsley, J.H., Kessler, D.J. and Hogan, M.E., Nucleic Acids Research, Vol.20: 307-314, 1992. Each of these is incorporated herein by reference.).

Please replace the paragraph (including heading) on page 49 line 16- line 28) with the following amended heading and paragraph:

Example V: Preparation of dFC GG ACG (SEQ ID NO: 7)

Oligonucleotides were synthesized by the phosphoramidite method cited above according to the methods described in Genome Research 7:741-747 (1998) which is hereby incorporated by reference. Preparation of oligonucleotides of the invention was carried out following per se known method utilizing the MerMade IV synthesizer (sold by BioAutomation, Texas USA). (The protocol of oligo synthesis is provided by the instrument manufacturer, and is used as outlined in the chart below). A typical example of preparation is given below taking preparation of dFC GG ACG (dFC == 2'-deoxy, 2',2'-difluorocytidine), as an example.

Preparation of the oligonucleotide dFC GG ACG (SEQ ID NO: 7)

Sequence : dFC GG ACG (SEQ ID NO: 7)

Synthesis scale: 10 μ mole

Please replace the paragraph on page 51, lines 7-8 with the following amended paragraph:

The desired ODN (oligodeoxynucleotide) sequence dFC GG ACG (SEQ ID NO: 7) was obtained at 90.6% purity as shown by HPLC analysis data (FIGURES 5A-B).

Please replace the paragraph on page 53 (lines 6-12, including the table) with the following:

Gemzar (MW 299.5), or 2'-deoxy, 2',2'-difluorocytidine hydrochloride for injection, as manufactured by Lilly France S.A., Fegersheim, France). Gemzar-ODNs were 2'-deoxy, 2',2'-difluorocytidine (dFC, or difluorodeoxycitidine) linked to 4 oligodeoxynucleotide (ODN) sequences (synthesized by Chemgenes Corp, Wilmington, MA) as follows:

DESIGNATION	GEMZAR-ODN (dFC=2'-deoxy 2',2'difluorocytidine hydrochloride)	MW	Quantity supplied	Gemzar equivalent
<u>Sequence-1</u> <u>SEQ ID NO: 7</u>	dFC GGA CG	1852.27	4002 nmole	193,805 µg
<u>Sequence-2</u> <u>SEQ ID NO: 8</u>	dFC GTG GAA CG	2798.87	5134 nmole	164,538 µg
<u>Sequence-3</u> <u>SEQ ID NO: 9</u>	dFC GGA CGT GGA ACG	4059.67	820 nmole	11,489 µg
<u>Sequence-4</u> <u>SEQ ID NO: 10</u>	dFC GGA GCT GGA ACG	4059.67	3450 nanomole	76,299 µg

Please replace the paragraphs and table from line 14 page 54 through line 12 page 55, with the following amended paragraphs, amended table(s) and new table(s):

FIG. 13 shows flow cytometric DNA cell cycle profiles showing 2'-deoxy, 2',2'-difluorocytidine -ODNs killing colon cancerous cells HT29 much more effectively than by treatment with Gemzar alone, at equivalent dosages. Cells were treated in culture with respective drugs for 1 hour at the stated dosage, and reincubated in normal medium without drugs for a further 47 hours. Column A is treatment by Gemzar alone. Column B-E are treatments by 2'-deoxy, 2',2'-difluorocytidine -ODN sequence 1-47-10, at equivalent Gemzar dosages.

DESIGNATION	GEMZAR-OLIGOS (dFC=GEMZAR)	MW	Quantity synthesized
<u>Sequence-1</u> <u>SEQ ID NO: 7</u>	dFC GGA CG	1852.27	233 OD = 4002 nmole
<u>Sequence-2</u> <u>SEQ ID NO: 8</u>	dFC GTG GAA CG	2798.87	457 OD = 5134 nmole
<u>Sequence-3</u> <u>SEQ ID NO: 9</u>	dFC GGA CGT GGA	4059.67	106 OD = 820 nmole

	ACG		
<u>Sequence 4</u> <u>SEQ ID NO:</u> <u>10</u>	dFC GGA GCT GGA ACG	4059.67	442 OD = 3450 nmole

In particular, FIG 13 (and FIG 14 described below) show data for the following:

<u>POSITION IN FIGURE</u>	<u>DATA FOR</u>
I(A)	<u>Control</u>
II(A)	<u>20ng/ml Gemzar</u>
II(B)	<u>20ng/ml Gemzar equivalent SEQ ID NO:7</u>
II(C)	<u>20ng/ml Gemzar equivalent SEQ ID NO:8</u>
II(D)	<u>20ng/ml Gemzar equivalent SEQ ID NO:9</u>
II(E)	<u>20ng/ml Gemzar equivalent SEQ ID NO:10</u>
III(A)	<u>50ng/ml Gemzar</u>
III(B)	<u>50ng/ml Gemzar equivalent SEQ ID NO:7</u>
III(C)	<u>50ng/ml Gemzar equivalent SEQ ID NO:8</u>
III(D)	<u>50ng/ml Gemzar equivalent SEQ ID NO:9</u>
III(E)	<u>50ng/ml Gemzar equivalent SEQ ID NO:10</u>

Figure 14 shows flow cytometric DNA cell cycle profiles showing 2'-deoxy, 2',2'-difluorocytidine -ODNs killing colon normal cells CCD-112CO more effectively than by treatment with Gemzar alone, at equivalent dosages. Cells were treated in culture with respective drugs for 1 hour at the stated dosage, and

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reincubated in normal medium without drugs for a further 47 hours. Column A is treatment by Gemzar alone. Column B-E are treatments by 2'-deoxy, 2',2'-difluorocytidine -ODN sequences 1-47-10, at equivalent Gemzar dosages, similar to those used in colon cancerous cells HT29 shown above in Figure 13.